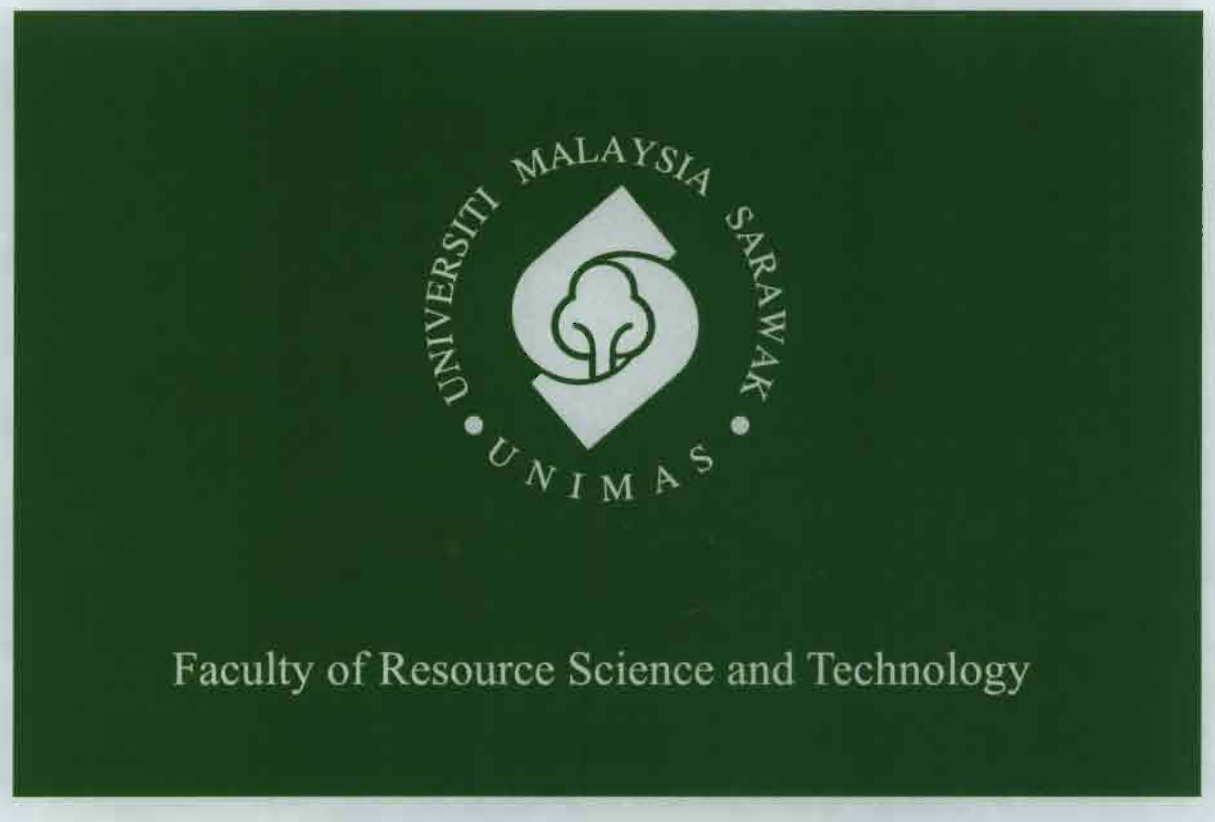

INVESTIGATION OF *ESCHERICHIA COLI, ESCHERICHIA COLI* O157:H7 AND *SALMONELLA TYPHIMURIUM* IN VARIOUS EXOTIC MEATS

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(39037)

Bachelor of Science with Honours
(Resource Biotechnology)
2015

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LIST OF ABBREVIATIONS

µl	Microliter
ml	Mililiter
bp	Base pair
CFU/ml	Colony forming unit per mililiter
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EC broth	*Escherichia coli* broth
E. coli	*Escherichia coli*
EMBA	Eosin-Methylene Blue agar
g	Gram
LB	Luria-Bertani
$MgCl_2$	Magnesium Chloride
min	Minute(s)
PCR	Polymerase Chain Reaction
s	Second
SCB	Selenite Cystein Broth
spp.	Species
S. typhimurium	*Salmonella typhimurium*
TBE	Tris-Borate-EDTA
UV	Ultra Violet
US	United States
V	Volt
XLD	Xylose Lysine Deoxycholate

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Investigation of *Escherichia coli*, *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Various Exotic Meats

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ABSTRACT

Escherichia coli (*E. coli*) O157:H7 and *Salmonella typhimurium* (*S. typhimurium*) are foodborne pathogens that cause food poisoning outbreaks since decades ago. Studies have shown that exotic meats have the potential to be contaminated by both pathogens. A total of 14 exotic meat samples namely wild boar (n=3), snake (n=3), soft-shelled turtle (n=3), frog (n=3), deer (n=1) and squirrel (n=1) were purchased and collected from wet markets, food court, restaurant and individuals for the present study. The isolation of *E. coli* and *Salmonella* spp. from the collected samples were accomplished through selective plating on EMBA and XLD agar respectively while polymerase chain reaction were used to detect the presence of *E. coli* O157:H7 and *S. typhimurium* in the collected samples. The findings showed that 100% (14/14) of the samples showed appearance of *E. coli* colonies on EMBA while 78.57% (11/14) of the samples showed appearance of *Salmonella* colonies on XLD agar. Both *E. coli* O157:H7 and *S. typhimurium* were not detected from the collected samples through polymerase chain reaction. However, the results cannot prove that exotic meats are safe to be consumed. Hence, more related studies are required to further assess the microbial risk on consuming exotic meats and ensure food safety.

Keywords: *Escherichia coli* O157:H7, *Salmonella typhimurium*, polymerase chain reaction, standard plate count, exotic meats

ABSTRAK

Escherichia coli (E. coli) O157:H7 dan Salmonella typhimurium (S. typhimurium) merupakan patogen bawaan makanan yang menyebabkan wabak keracunan makanan sejak beberapa dekad yang lalu. Kajian telah menunjukkan bahawa daging eksotik berpotensi untuk dicemarkan oleh kedua-dua patogen tersebut. Sebanyak 14 sampel daging eksotik iaitu babi hutan (n=3), ular (n=3), labi-labi (n=3), katak (n=3), rusa (n=1) dan tupai (n=1) telah dibeli dan dikumpul dari beberapa pasar basah, medan selera, restauran dan beberapa individu untuk kajian ini. Pengasingan E. coli dan spesies Salmonella dari sampel-sampel yang dikumpul dapat dicapai dengan menggunakan selective plating di atas EMBA dan agar XLD masing-masing manakala polymerase chain reaction digunakan untuk mengesan kewujudan E. coli O157:H7 dan S. typhimurium dalam sampel-sampel yang dikumpul. Penemuan kajian ini menunjukkan bahawa 100% (14/14) sampel menunjukkan kemunculan koloni-koloni E. coli di atas EMBA manakala 78.57% (11/14) sampel menunjukkan kemunculan koloni-koloni Salmonella di atas agar XLD. Kedua-dua E. coli O157:H7 dan S. typhimurium tidak dikesan dari sampel-sampel yang dikumpul dengan menggunakan polymerase chain reaction. Namun, keputusan tersebut tidak dapat membuktikan bahawa daging eksotik adalah selamat untuk dimakan. Oleh itu, kajian-kajian yang berkenaan amat diperlukan untuk menilai risiko mikrob dalam pengambilan daging eksotik dan memastikan keselamatan makanan.

Kata kunci: *Escherichia coli*, *Salmonella typhimurium*, polymerase chain reaction, standard plate count, daging eksotik

1.0 INTRODUCTION

Since decades ago, foodborne diseases have become global burden which are mainly due to contamination of food and drinking water by foodborne pathogens. Newell *et al.* (2010) mentioned that foodborne diseases are not just an underdeveloped countries problem as about 76 million cases of foodborne illness are estimated to arise each year in USA, resulting in 325000 hospitalizations and 5000 deaths. *Escherichia coli* O157:H7 and *Salmonella typhimurium* are the examples of common foodborne pathogens.

In 1982, *E. coli* O157:H7 was first recorded as foodborne pathogen during an investigation of hemorrhagic colitis outbreak due to the consumption of hamburgers from a fast food restaurant (Marler Clark, 2015). A recent outbreak of *E. coli* O157:H7 in Oregon was linked to the consumption of strawberries contaminated by feces of deer, resulting in 6 cases of hospitalization with 2 deaths from hemolytic uremic syndrome (Laidler *et al.*, 2013). An outbreak of *S. typhimurium* DT 104 associated with the consumption of bakery products such as sausage rolls, pies and sandwiches from a local bakery and retail chain was reported in Tayside in 1996 (Roworth *et al.,* 1997). In 2013, there was a multistate *S. typhimurium* outbreak in U. S. due to the live poultry in backyard flocks which results in 62 cases of hospitalization (Center for Disease Control and Prevention, 2013). Although there is no report of large outbreak of *E. coli* O157:H7 and *S. typhimurium* in Malaysia, various studies and investigation on the potential sources of contamination still need to be carried out to prevent the outbreaks of both pathogens.

Consumptions of poultry meat or beef were often reported as the causes of *E. coli* O157:H7 and *S. typhimurium* outbreaks (Adeyanju and Ishola, 2014; Center for Disease Control and Prevention, 2014; Kivi *et al.,* 2007; Marler Clark, 2015). However, exotic meats such as wild boar, snake, soft-shelled turtle, frog, deer and squirrel that consumed by

Malaysian especially Sarawakian can be the potential reservoirs for *E. coli* O157:H7 and *S. typhimurium*. *E. coli* O157:H7 has been isolated from feces sample of wild boar in southwest Spain (Sánchez *et al.*, 2010). In a study by Angulo *et al.* (2010), reptiles particularly turtle are considered as unsafe pet to children due to the *Salmonella* hidden on their skin, shell or feces. Hoelzer *et al.* (2011) mentioned that birds, rodents, reptiles, amphibians, exotic pets and wild life can be the potential reservoirs for *Salmonella*. Hence, it is believed that exotic meats are at a high risk of being contaminated by *E. coli* O157:H7 and *S. tyhimurium* based on the findings from previous studies. In order to ensure food safety, more related studies are required to assess and investigate the presence of *E. coli* O157:H7 and *S. typhimurium* on different types of exotic meats as there are lack of related studies in Malaysia.

Therefore, this study was undertaken with the following objectives:

i. To enumerate *E. coli, E. coli* O157:H7 and *S. typhimurium* in exotic meats using Standard Plate Count method.

ii. To detect the presence of *E. coli*, *E. coli* O157:H7 and *S. typhimurium* in exotic meats using Polymerase Chain Reaction (PCR) assay.

iii. To analyze and compare the presence of *E. coli*, *E. coli* O157:H7 and *S. typhimurium* in different types of exotic meats.

2.0 LITERATURE REVIEW

2.1 Characteristics of *Escherichia coli* O157:H7 and *Salmonella typhimurium*

In 1885, *Escherichia coli*, a Gram negative, rod-shaped facultative anaerobic bacterium, was first identified by Theodor Escherich (Lim *et al.*, 2010). It is a member of the family *Enterobacteriaceae* (Frederick, 2011). Most *E. coli* strains are harmless but some strains have evolved into pathogenic *E. coli* which can cause severe diseases to human. Mead and Griffin (1998) stated that *E. coli* O157:H7 is one of the pathogenic *E. coli* that can produce Shiga toxins. Due to the expression of somatic (O) antigen 157 and flagella (H) antigen 7, it is named as *E. coli* O157:H7. The organisms are able to survive in various environments such as water, soil, food and animal reservoirs as they can grow and survive at temperature ranging from 7°C to 50°C with an optimum temperature of 37°C (Lim *et al.*, 2010; World Health Organization, 2011). Furthermore, they are resistant to acid and thus they are able to survive in the acidic environment of the stomach, increasing the possibilities of the bacteria to invade the intestines and cause diseases (Lim *et al.*, 2010).

Ido *et al.* (2014) stated that *Salmonella* species are subdivided to many serovars through serotyping and *Salmonella typhimurium* was recognized as one of the serovars of *Salmonella enterica* based on the White-Kaufmann-Le Minor scheme. *Salmonella* spp. are Gram negative, rod in shape and facultative anaerobic (D' Aoust, 1997). They belong to the *Enterobacteriaceae* family (Public Health Agency of Canada, 2011). The bacteria are able to survive and grow well at temperature ranging from 2°C to 54°C within a pH range of 4.5 to 9.5. Moreover, they are motile with the presence of flagella, a tail-liked projection made of proteins (Chandler, 2011).

2.2 Occurrence of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in Food

Several studies have been conducted on the prevalence, isolation and detection of *Escherichia coli* O157:H7 in food. Rahimi *et al.* (2012) have isolated and detected *E. coli* O157:H7 from 1 sample of 201 dairy products purchased from supermarkets and retail shops in several provinces of Iran. In another study by Daube *et al.* (2013) in Lubumbashi, 81.3% of 182 samples of smoked game meat from three species sold at retail outlets were found to be contaminated by *E. coli*. *E. coli* O157:H7 was also found and identified in feces, hides and carcasses of meat goats from U.S. processing plant with prevalence of 11.1%, 2.7% and 2.7% respectively (Jacob *et al.*, 2013). Chang *et al.* (2013) also detected *E. coli* O157:H7 in 5.2% of 230 organic food samples collected including tomato, lettuce, red cabbage, white radish, cucumber, chinese cabbage, four-winged bean and chicken from supermarkets and retail groceries in Malaysia.

There were also many previous studies carried out to isolate and detect *Salmonella typhmurium* from various food samples. In a study by Jamshidi *et al.* (2009), *S. typhimurium* has been detected in 1.6% of poultry carcasses sampled from a commercial broiler slaughtering facility in Mashhad-Iran. *S. typhimurium* was also detected in the samples of raw salad vegetables and vegetarian burger patties from markets in Selangor state of Malaysia with a prevalence of 14.3% in a study conducted by Nillian *et al.* (2011). Adeyanju and Ishola (2014) found that 33% of 99 poultry samples collected from retail markets in Oyo state of Nigeria was contaminated by *Salmonella* spp. *Salmonella* spp. was also detected in 182 samples of smoked game meats from retail outlets in Lubumbashi with a culture prevalence of 4.3% (Daube *et al.*, 2013).

2.3 Transmission and Diseases

According to Lim *et al.* (2010), consumption of contaminated food and water is the most common transmission route for *Escherichia coli* O157:H7 infection. Cattle are the main reservoir for *E. coli* O157:H7 as contaminated beef products are often reported as the causes of *E. coli* O157:H7 outbreaks. The pathogen may be introduced into the meat during the process of slaughtering or grinding (Mead and Griffin, 1998). Besides, it can also be transmitted directly from animal to people and from person to person especially in child day-care facilities (Lim *et al.*, 2010). Tidy (2014) found that animal keepers, farmers and farm visitors are the potential victims that may be infected by the pathogen when they are in contact with the farm animals. Waterborne transmission of *E. coli* O157:H7 can occur via drinking or swimming in unchlorinated water (Mead and Griffin, 1998). Most cases of *E. coli* O157:H7 infections begin with abdominal cramps and non-bloody diarrhea and the infected person can recover from the infection without further complication (Lim *et al.*, 2010). However in some cases, the disease will develop into bloody diarrhea or hemorrhagic colitis and even progress to the life-threatening sequelae, hemolytic uremic syndrome (HUS) or thrombocytopenic purpura (Lim *et al.*, 2010). HUS can be a fatal disease as it leads to the destruction of red blood cells and kidney failure (New York Department of Health, 2006).

Generally, *Salmonella* bacteria are transmitted through the fecal-oral route whereby the food and drinks consumed have been contaminated by the pathogen-containing fecal matter (Chandler, 2011). Besides, they are zoonotic and thus they can be transferred from animals to human. In this case, human can be infected by the pathogen through the contacts with pets such as cats, dogs, birds and turtles as they are the normal host of the bacteria (World Health Organization, 2013). Public Health Agency of Canada (2011) reported that person-to-person contact can also be a mode of transmission of the pathogen.

For example, in the pediatric wards of hospitals, the pathogen can be transmitted from one person to another person through their hands or improperly disinfected scope. Infection by *Salmonella* bacteria can lead to salmonellosis with symptoms such as diarrhea, nausea, fever and abdominal pain (World Health Organization, 2013). Gastroenteritis, also known as food poisoning, is mainly caused by the infection of *Salmonella typhimurium* with similar symptoms as salmonellosis (Public Health Agency of Canada, 2011). In most cases, the patients are able to recover from the infections without any specific treatment (World Health Organization, 2013). However, the disease can be severe and fatal especially to children and elders due to dehydration.

2.4 Treatment and Prevention

Vermont Department of Health (2008) mentioned that antibiotics are not encouraged to be given to patients as treatment for *Escherichia coli* O157:H7 infections as some antibiotics can cause harm to kidneys. Furthermore, the use of antibiotics as treatment is believed to increase the severity of the disease due to the toxins released by damaged bacteria (Davis, 2015). Hence, hydration is the main treatment used to avoid further complication caused by dehydration, either in oral form or through intravenous hydration. There are several precautions that can be taken to prevent the infection of *E. coli* O157:H7. In the aspect of food preparation, raw meat must be kept away from ready-to-eat food to prevent cross contamination (Vermont Department of Health, 2008). All fruits and vegetables that are eaten raw must be washed properly and meat must be cooked thoroughly before being served (Tidy, 2014). Raw or unpasteurized milk and dairy products should also be avoided. Moreover, proper hand washing procedure must be practiced before having meal, after using toilet, handling raw meat and contact with animals. In addition, avoid drinking or swimming in water that may be contaminated.

For mild or moderate cases of *Salmonella* infection, antimicrobial treatment is also not recommended for healthy individuals but antibiotics such as ampicillin, cephalosporin and ciproflaxin may be required to treat children, elderly and immunocompromised groups (Public Health Agency of Canada, 2011). Klochko (2014) mentioned that in severe cases, fluid and electrolyte treatment are applied to control the symptoms of vomiting and diarrhea. In the case of prevention, control measures are required at every stage of food chain from agricultural production to food preparation. In the field, the fecal wastes used for growing fruits and vegetables must be treated first before they are being applied (World Health Organization, 2013). Besides, the harvest and storage equipment should always be clean and dry. For food preparation, the prevention steps are similar to the prevention steps of *E. coli* O157:H7 infection. Parents or adults must supervise their children carefully when they are in contact with pet animals as the pathogen can be transmitted directly from animals to children (World Health Organization, 2013). Good personal hygiene must also be practiced at anytime and anywhere to prevent infection by the pathogen.

2.5 Polymerase Chain Reaction

In 1983, polymerase chain reaction was first developed by Kary Mullis (Rahman *et al.*, 2013). Now, PCR is the most widely used molecular biology technique for the amplification of small amount of DNA molecules in an exponential manner. Rahman *et al.* (2013) stated that PCR has been applied in various fields to accomplish many tasks such as paternity testing, detection of hereditary diseases, cloning of genes and DNA computing. Furthermore, the development of PCR also greatly contributes to gene analysis, diagnosis of diseases and the detection of microorganisms (Valones *et al.*, 2009). PCR is a sensitive technique as targeted sequences are able to be detected from small amount of DNA (Patricia and Arlen, 2012). Moreover, specificity is one of the advantages of PCR whereby it is conducted under specific conditions. In detecting microorganisms, PCR is considered

a fast approach compared to other means with complicated procedures (Patricia and Arlen, 2012). The versatility of PCR enables the identification of genetic sequences from different microorganisms for diagnosis of different pathologies using the same reaction conditions. In order to enhance performance and specificity, the basic PCR method has been modified and variants have been developed. Multiplex PCR is one of the variants developed in which several DNA sequences can be amplified simultaneously (Patricia and Arlen, 2012). In addition, different pathogen can be detected at the same time in a single sample through multiplex PCR.

In previous studies, PCR have been used for the detection of *E. coli* O157:H7 and *S. typhimurium* in different types of food. Ibrahim *et al.* (2014) detected the presence of *E. coli* O157:H7 and *S. typhimurium* in lettuces and carrots collected from Egyptian farms using PCR. *E. coli* O157:H7 was also detected in organic vegetables and chickens through the application of PCR in a study conducted by Chang *et al.* (2013). Furthermore, multiplex PCR has been used to detect the presence of *S. typhimurium* in raw salad vegetables and vegetarian burger patties by Nillian *et al.* (2011).

3.0 MATERIALS AND METHOD

The materials used in this study are listed in Appendix I.

3.1 Sample Collection

A total of 14 raw exotic meat samples including wild boar, snake, frog, soft-shelled turtle, deer and squirrel were collected from individuals or purchased from the 7th mile Wet Market, Serian Wet Market, Siang Siang Food Court and restaurant as shown in Table 1. Samples were collected in sterile plastic bags and processed immediately upon arrival to the laboratory.

Table 1. Types, sources and number of samples.

Samples	Sources	No. of Samples
Wild Boar	7th mile wet market & individuals	3
Snake	Serian wet market & individual	3
Frog	Siang Siang Food Court	3
Soft-shelled Turtle	Serian wet market	3
Deer	Restaurant	1
Squirrel	Individual	1
	Total	14

3.2 Processing and Enrichment of Samples

Twenty five grams of the sample was added with 225 ml of *Escherichia coli* broth (EC broth; Oxoid, UK) and homogenized in a stomacher bag. Similarly, 25 g of the sample was added into 225 ml of Selenite Cystein Broth (SCB; Oxoid, UK). The samples were incubated at 37 °C for 18 to 24 hours. The pre-enriched samples were diluted for ten-fold serial dilutions from 10^{-1} to 10^{-7} dilution.

3.3 Standard Plate Count

Aliquot of 0.1 ml of each dilution from EC broth and SCB were spread plated on the surface of Eosin-Methylene Blue agar (EMBA; Oxoid, UK) and Xylose Lysine Deoxycholate (XLD; Oxoid, UK) agar respectively (Faith *et al.,* 1996). Spread plates were performed in duplicate for each dilution. The plates was inverted and incubated for 18 to 24 hours at 37 °C. After incubation, results were observed and the colonies formed were counted and recorded.

3.4 Preparation of Stock Culture

Three colonies were picked from EMBA plate and XLD agar plate and transferred to Luria-Bertani (LB) nutrient agar slants. The slant agars were kept as stock culture of the bacteria. For *E. coli*, a green metallic sheen was picked from the EMBA while for *S. typhimurium*, red colony with blacken center was picked from XLD agar (Murray, 1996). The inoculated tubes were then incubated for 18 to 24 hours at 37 °C.

3.5 DNA Extraction

Deoxyribonucleic acid (DNA) extraction was carried out using boil cell method as described by Chang *et al.* (2013) with some modification. Bacterial colonies selected from both agars for each dilution was transferred to 100 µl of sterile distilled water and boiled

for 10 min before cooling at -20 °C for another 10 min. The cooled mixture was centrifuged at 12000 xg for 5 min. The supernatant was used as template for Polymerase Chain Reaction (PCR) amplification.

3.6 PCR Amplification

For the detection of *E. coli* O157:H7 and *S. typhimurium*, multiplex-PCR assay was carried out according to Kasing *et al.* (2011) and Nillian *et al.* (2011) respectively. A total of 25 µl reaction mixtures containing 1x PCR buffer, 0.5 mM deoxynucleoside triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 1 U of *Taq* polymerase and 5 µl of DNA for *E. coli* or 2 µl of DNA for *Salmonella* spp. were used to perform PCR amplification (Loo *et al.*, 2013). The sequence of the primer pairs used for the detection of *E. coli* O157:H7 and *S. typhimurium* were shown in Table 2. For the detection of *Stx1, Stx2, fliC*$_{h7}$ and *rfbE* genes of *E. coli* O157:H7, the amplification was performed as follow: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 1 min, and elongation at 72 °C for 1 min and a final extension at 72 °C for 7 min at the end of the process (Kasing *et al.*, 2011). For the detection of *fliC* gene of *S. typhimurium*, the amplification was performed using the same parameters as the amplification of *E. coli* O157:H7 genes (Nillian *et al.*, 2011).

Table 2. Primer pairs of *E. coli* O157:H7 and *S. typhimurium*.

Species	Primers	Sequence (5' to 3')	Target gene	Amplicon size (bp)	Reference
E. coli O157:H7	SltI-F	TGT AAC TGG AAA GGT GGA GTA TAC	*stx1*	210	Meng *et al.*, 1997
	SltI-R	GCT ATT CTG AGT CAA AAA ATA AC			
	SltII-F	GTT TTT CTT CGG TAT CCT ATT CCG	*stx2*	484	Meng *et al.*, 1997
	SltII-R	GAT GCA TCT CTG GTC ATT GTA TTA C			
	$FLIC_{h7}$-F	GCG CTG TCG AGT TCT ATC GAG C	$fliC_{h7}$	625	Gannon *et al.*, 1997
	$FLIC_{h7}$-R	CAA CGG TGA CTT ATC GCC ATT CC			
	Rfb-F	GTG TCC ATT TAT ACG GAC ATC CAT G	*rfbE*	292	Gannon *et al.*, 1997
	Rfb-R	CCT ATA ACG TCA TGC CAA TAT TGC C			
S. typhimurium	Fli 15-F	GGG TGT TGC CCA GGT TGG TAA T	*fliC*	559	Jamshidi *et al.*, 2009
	Tym-R	GCTGCAACTGTTACAGGATATGCC			

3.7 Agarose Gel Electrophoresis

After amplification, the PCR products was loaded onto 1.5% agarose gel and undergo electrophoresis at 90 V for 60 min in the presence of 1x Tris-Borate-EDTA (TBE) buffer (Loo *et al.*, 2013). One hundred bp and 1 kb DNA ladder were used as the marker. PCR products were visualized under Ultra Violet (UV) transilluminator after staining with ethidium bromide using gel documentation (WiseUV®, Germany). The expected sizes of amplicons for *stx1*, *stx2*, *flic*$_{h7}$, *rfbE* and *fliC* genes were 210, 484, 625, 292 and 559 bp respectively.